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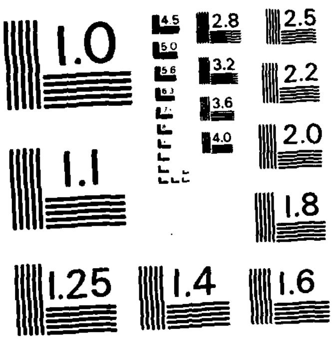

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Annual Report

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Enzymatic Conversion of Red Cells for Transfusion

Jack Goldstein, Ph.D., Principal Investigator
Jong-Yuan Kuo, Ph.D., Research Fellow
Leslie Lenny, Graduate Student
Geraldine Siviglia, Sr. Research Technician

NEW YORK BLOOD CENTER
NEW YORK, NY

Characterization of an Exo(α-)glycosidase Attached to a Soluble Support.

We have co-valently linked an α-galactosidase from green coffee beans to Dextran T-70 (weight average molecular weight of 70,000) under conditions designed to maximuze the amount of protein bound while minimizing loss of activity. Activation of dextran is carried out at a ratio of 1 molecule of cyanogen bromide per 17 glucose residues to minimize the crosslinking of the polymer and to ensure a medium free of unreacted cyanogen bromide when the enzyme is added. Also, to protect the active site of the enzyme during coupling at pH 8.4, a saturating level of D-galactose is included in the reaction mixture. Following coupling, the enzyme conjugate is routinely separated from unbound enzyme by filtration through Sephadex (Fig. 1) and monitored for purity by sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis as shown in Figure 2. Further evidence of covalent attachment of the enzyme to dextran was provided by treatment of the putative enzyme-dextran conjugate with dextranase which resulted in the conversion of the high molecular weight region to one resembling free α-galactosidase (Fig. 2C). The digestion product showed an increase in enzyme activity as well (Table I), suggesting that the lower activity of the enzyme conjugate following coupling is not due to irreversible denaturation of the enzyme but rather to inaccessibility of the substrate. Furthermore, the unreacted enzyme recovered from the column in the second elution region had essentially the same specific activity as it did prior to coupling, indicating that no denaturation had occurred under our reaction conditions. These enzyme coupling and separation conditions have resulted in an α-galactosidase-dextran conjugate completely separated from unbound enzyme and containing 80-90% of the starting free enzyme protein while retaining 50-55% of its specific activity. This is a considerable increase in activity yield from the 20% we obtained in our initial experiments.

The column fractions containing the α-galactosidase-dextran conjugate can be either concentrated using Amicon YM-10 membranes or dialyzed against distilled water and lyophilized without significant loss of activity. No evidence of free enzyme release was found when the lyophilized conjugate was reconstituted and examined by gel electrophoresis before and after using several times over a period of at least one month. During this time reuse and relyophilization did not produce any significant loss of activity.

The patterns of heat inactivation for free enzyme in the absence and presence of added dextran and enzyme-dextran conjugate are shown in Figure 3. The free enzyme lost only about 10% of its activity up to 45°, but rapidly declined in activity thereafter with a 20% loss at 50°, 70 to 75% at 55°, and complete inactivation at 60°. The addition of dextran to the free enzyme exerted only a slightly protective effect. However, the enzyme conjugate was more resistant to heat inactivation over a wider range of temperature than the free enzyme, showing only a 5% loss of activity up to 50°, a 15% reduction at 55° and was not completely inactivated until 65°. Other experiments revealed that the rate of thermal inactivation of free enzyme was markedly faster than for the enzyme conjugate. Even at temperatures where the free

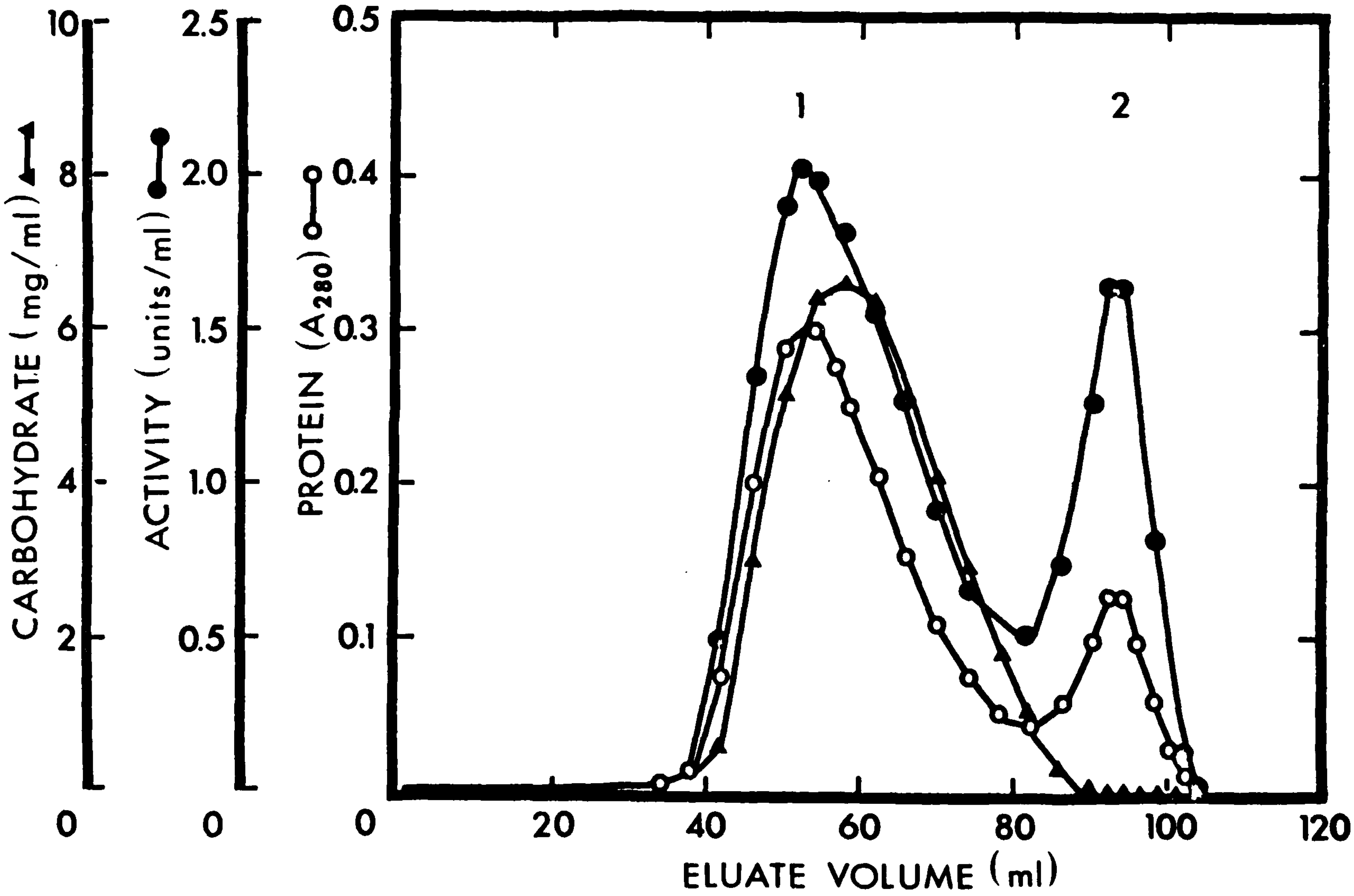
Fig. 1. Elution Profile of the α-Galactosidase-Dextran Reaction Mixtures by Sephadex G-200 Chromatography.

(1) Enzyme-dextran conjugate

(2) Free enzyme

The carbohydrate component was distributed only in the conjugate fractions. The conjugate contained 24.3 mg of enzyme per g of dextran which represents 80% of the starting protein retaining 55% of the original specific activity.

Figure 1



CARBOHYDRATE (mg/ml)
ACTIVITY (units/ml)
PROTEIN (A_{280})
10
8
6
4
2
0
2.5
2.0
1.5
1.0
0.5
0
0.5
0.4
0.3
0.2
0.1
0
1
2
20
40
60
80
100
120
ELUATE VOLUME (ml)

FIG. 2 SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORETIC PATTERNS.

(A) ENZYME-DEXTRAN CONJUGATE - ELUTION REGION 1.

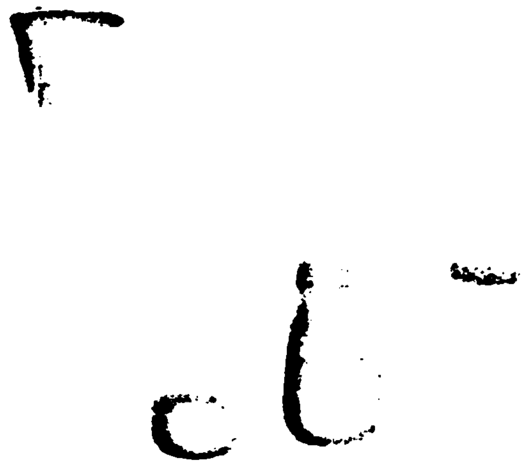
(B) FREE ENZYME - ELUTION REGION 2.

(C) CONJUGATE TREATED WITH DEXTRANASE.

(D) DEXTRANASE.

THESE RESULTS PROVIDE EVIDENCE FOR COVALENT ATTACHMENT OF THE ENZYME TO DEXTRAN. THE ENZYME-DEXTRAN CONJUGATE IS A HIGH MOLECULAR WEIGHT, POLYDISPERSE MIXTURE AND CAN BE DIGESTED TO THE FREE ENZYME BY DEXTRANASE TREATMENT.

FIGURE 2



(A) (B) (C) (D)

TABLE I. ENZYME ACTIVITY[A] OF CONJUGATE AND FREE ENZYME BEFORE AND AFTER DEXTRANASE DIGESTION

SAMPLES	ASSAY CONDITION 1.25 mM [S] pH 6.5	0.75 mM [S] pH 5.8	0.5 mM [S] pH 5.8
1. α-GALACTOSIDASE-DEXTRAN	0.39	0.71	0.77[B]
2. α-GALACTOSIDASE-DEXTRAN +DEXTRANASE	1.03[B]	0.93	0.89
3. α-GALACTOSIDASE[C]	0.59	0.47	0.45

A. EXPRESSED AS UNITS/ML

B. 34% INCREASE IN ACTIVITY FOLLOWING DEXTRANASE DIGESTION.

C. VALUES ARE THE SAME WITH OR WITHOUT DEXTRANASE.

THE 34% INCREASE IN ACTIVITY FOLLOWING DEXTRANASE TREATMENT OF THE ENZYME CONJUGATE SUGGESTS THAT THE PRIMARY LOSS OF ACTIVITY FOLLOWING COUPLING IS NOT DUE TO IRREVERSIBLE DENATURATION OF THE ENZYME BUT RATHER TO RESTRICTIONS IMPOSED UPON IT. THE TREATMENT ALSO RESULTED IN A CONVERSION OF THE OPTIMAL SUBSTRATE CONCENTRATION BACK TO THAT FOR THE FREE ENZYME.

FIG. 3. EFFECT OF TEMPERATURE UPON FREE AND BOUND ENZYMES AFTER INCUBATION FOR 15 MINUTES AT pH 6.5 AND 5.8 RESPECTIVELY.

•—• ENZYME CONJUGATE

△—△ ENZYME + DEXTRAN

○—○ ENZYME

THE ENZYME CONJUGATE WAS MORE RESISTANT TO HEAT INACTIVATION THAN TO FREE ENZYME. THE ADDITION OF DEXTRAN TO THE FREE ENZYME EXERTED ONLY A SLIGHTLY PROTECTIVE EFFECT.

FIGURE 3

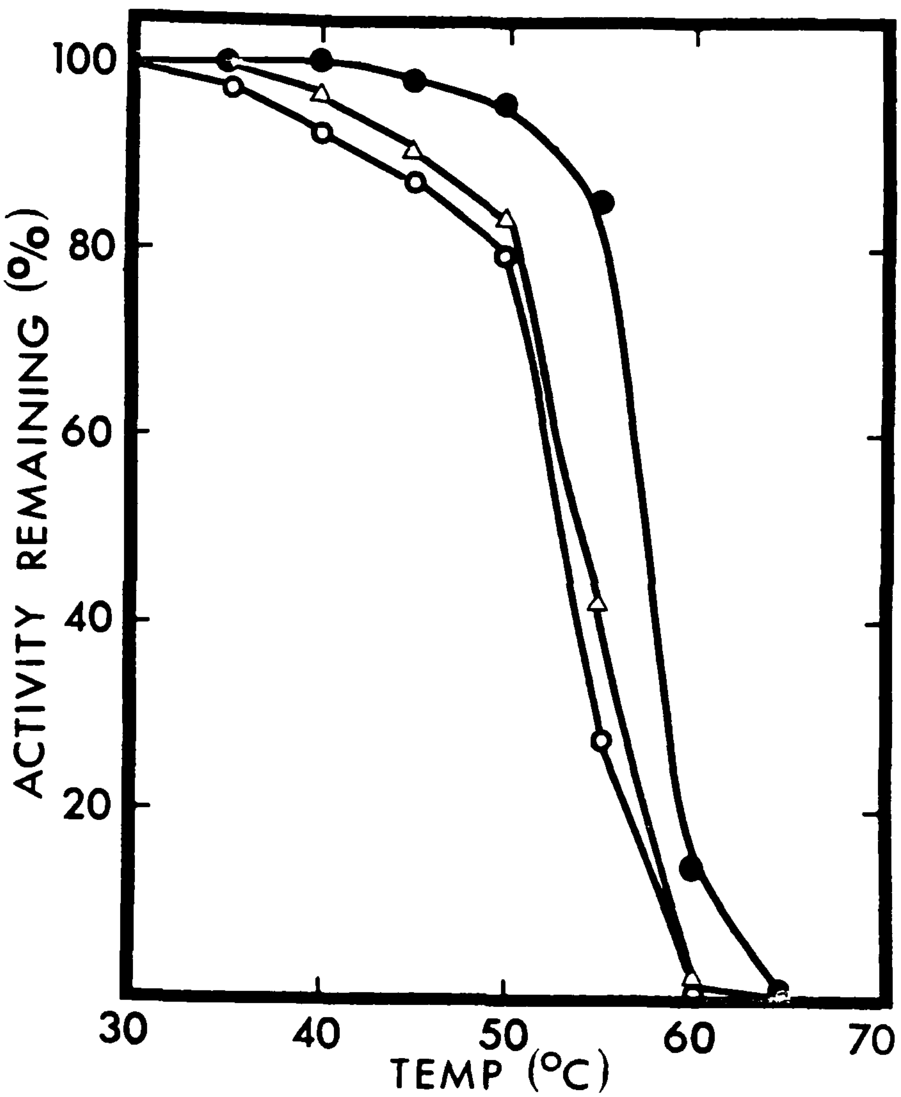


FIG. 4 pH PROFILES FOR HYDROLYSIS OF p-NITROPHENYL α-D-GALACTOSIDE CATALYZED BY THE

(A) ENZYME-DEXTRAN CONJUGATE

(B) FREE ENZYME

AT THE INDICATED SUBSTRATE CONCENTRATION.

THE pH OPTIMUM AT 5.6 IS NOT SUBSTRATE CONCENTRATION DEPENDENT FOR THE CONJUGATE, IN CONTRAST TO THE [S] DEPENDENCY OF FREE ENZYME.

FIGURE 4

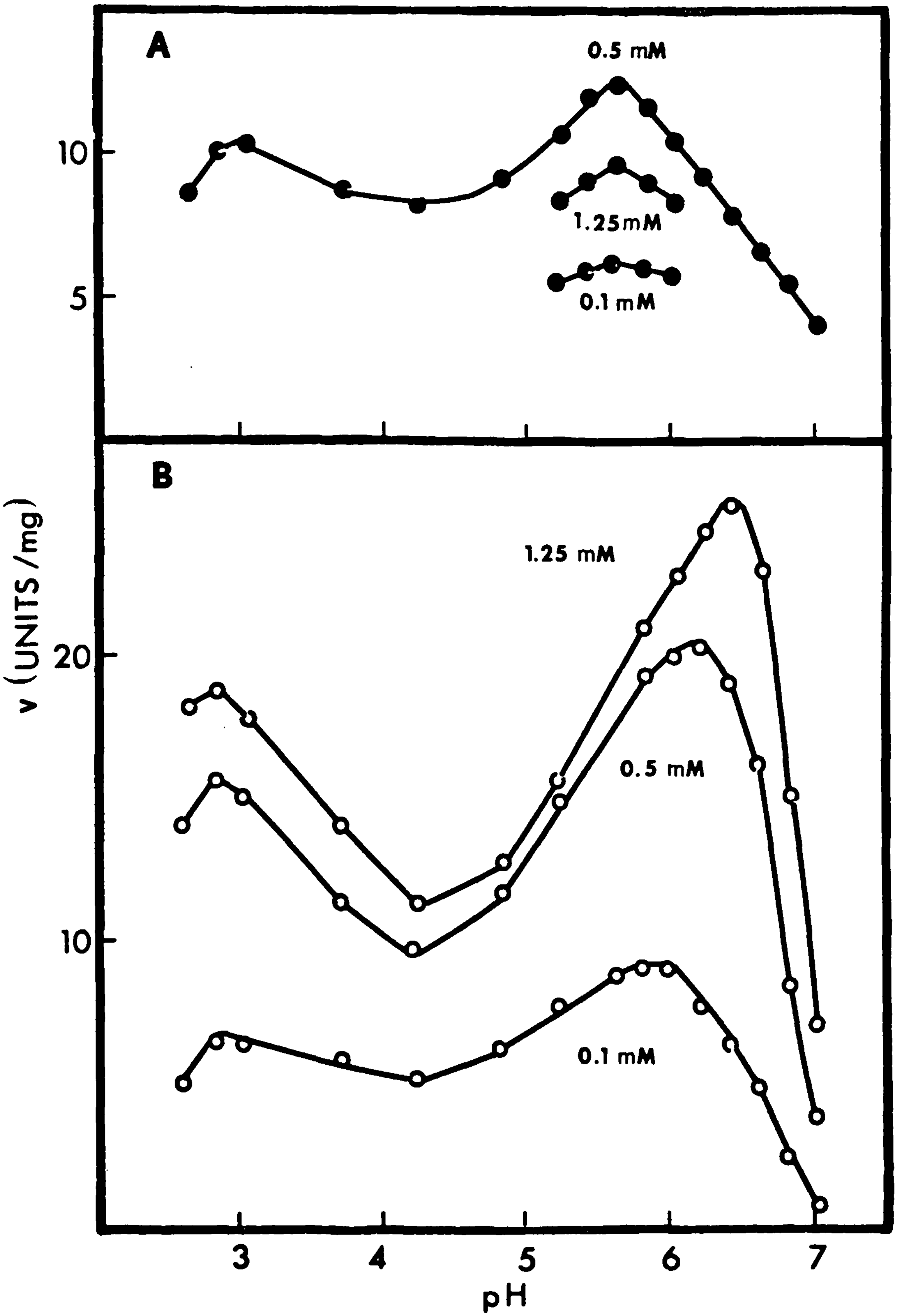

A
0.5 mM
10
5
1.25 mM
0.1 mM
B
1.25 mM
20
v (UNITS/mg)
0.5 mM
10
0.1 mM
3
4
5
6
7
pH

TABLE II. KINETIC CONSTANTS FOR α-GALACTOSIDASE AND α-GALACTOSIDASE-DEXTRAN

ALL ASSAYS WERE PERFORMED AT 26° IN 0.1 M POTASSIUM PHOSPHATE BUFFER.

	α-GALACTOSIDASE		α-GALACTOSIDASE-DEXTRAN
	pH 6.5	pH 5.8	pH 5.8
K_M (mM)	0.33	0.16	0.09
K_S (mM	4.7	3.7	3.1
K_I (mM)	0.18	0.16	0.13
V_{MAX} (UNITS · MG^{-1})	34	27	13
V_{MAX}/K_M (UNITS · MG^{-1} · mM^{-1})	103	169	144

ALL THE CONSTANTS ARE LOWER FOR THE CONJUGATE. THE RESULTANT V_{MAX}/K_M OF THE CONJUGATE IS NOT MUCH LOWER THAN THAT OF FREE ENZYME AT pH 5.8.

enzyme was initially stable such as 37°, 20% of its activity was lost after six hours as compared with no loss of activity for the enzyme conjugate.

The pH activity profiles of the immobilized and free enzymes have been determined by measuring the initial rate of hydrolysis of the synthetic substrate p-nitrophenyl-α-D-galactoside (Fig. 4). The free enzyme was found to have two pH optima: the minor in the acidic region at pH 2.8-3.0 and the major which is substrate concentration dependent peaking at pH 6.4, 6.1 and 5.9 at substrate concentrations of 1.25, 0.5 and 0.1 mM respectively. The enzyme dextran conjugate yielded a somewhat different profile. The pH maximum in the acid region was unchanged but the other which is not substrate concentration dependent had shifted to pH 5.6 and had become broader and relatively less pronounced. Increases of ionic strength with phosphate, citrate and sodium chloride had no effect on this pH shift. However, dextranolytic digestion of the conjugate shifted the pH optimum back to that of the free enzyme (Table I).

Kinetic studies show that the free enzyme exhibits an optimal substrate concentration of 1.25 mM at pH 6.5 and 0.75 mM at pH 5.8 whereas that of the enzyme conjugate shifts to 0.5 mM at both of these pHs. D-galactose was found to be a competitive inhibitor of both free and bound enzymes. As shown in Table II, α-galactosidase-dextran's K_m, K_s, K_I and V_{max} are all smaller than free enzyme even when the constants for free enzyme are determined at pH 5.8 instead of 6.5.

As mentioned in my status report, in order to increase the amount of enzyme activity bound per mg of dextran which in the case of T-70 is approximately 0.3 units, we are now attaching enzyme to dextran having an average molecular weight of 40,000 (T-40) and obtaining about 0.5 units per mg dextran. Recent studies with such preparations using p-nitrophenyl-α-D-galactoside has shown them to have the same pH optima, thermal stability and very similar kinetic properties as the T-70 enzyme-dextran conjugate. Both types of enzyme conjugates have the same specificity as free enzyme for the removal of the B antigenic determinant from the surface of the red cell. In view of the small difference in V_{max}/K_m values (Table II) between the free and bound enzymes at pH 5.8, no significant reduction in kinetics would be expected when red cells are treated with the latter. Also, substrate inhibition would not occur since the amount of terminal D-galactose released by enzyme treatment is in the order of 0.06 μmole per ml of packed red cells. Results obtained thus far under our current conversion conditions bear this out since they show the conjugates to have kinetics at least as fast as those of free enzyme for the removal of B antigenic activity from the surface of red cells. The enzyme-dextran conjugates also have the advantages of increased thermal stability, and of a pH optimum in the region found to facilitate antigen removal from the red cell. They can be reused and, unlike free enzyme, minimize the possibility of protein contamination of the red cell following enzymatic treatment.

We have begun to isolate and examine α-N-acetylgalactosaminidases in order to find one which will remove A antigenic activity from the red cell so that we can attach it to dextran and study its properties. Of the three thus far reported to be active with blood group substances we have been studying the one obtainable from Aspergillus niger. We were able to obtain partially purified enzyme preparations free of si..lidase activity. However, they could not remove A antigenicity from red cells under our conversion conditions, probably because the effective pH of the enzyme is in the highly acid region (pH 4.6). We plan to continue and intensify our search for an appropriate α-N-acetylgalactosaminidase from other sources.

Structural and Metabolic Properties of Enzymatically Converted Type B Red Cells.

We have been able to develop antibodies in rabbits against free α-galactosidase and α-galactosidase dextran (prepared from Dextran T-70). The results of immunodiffision studies which measure the titer of precipitating antibodies show that the higher titer antibody preparation is the one developed against the enzyme-dextran conjugate. This was expected since protein-dext.an conjugates have been shown to be strongly immunogenic in rabbits. We have begun using both these antibody systems as a probe for determining if any of the components of the enzyme preparations remain attached to the red cell surface following treatment by free enzyme or enzyme-dextran. Preliminary results have been negative. We are also labeling IgG antibody mixtures containing anti B with radioactive iodine [I^{125}]. After further purification we will use these labeled antibodies in an attempt to determine if any B antigenic sites remain on the red cells following enzymatic conversion.

Our current treatment conditions for the enzymatic removal of B antigenicity involve incubating a 10% suspension of red cells of pH 5.8 in a buffer consisting of either citrate-phosphate or only phosphate in isotonic saline at 26° using an end-over-end mixer rotating at 2-5 r.p.m. Following incubation, the treated cells are washed several times using phosphate buffered saline at pH 7.4 and allowed to "recover" i.e. become adjusted to the pH of this buffer by remaining in it for 30 min. before being used for structural or metabolic studies. Since the pH optima for the free and bound enzymes are in the acid region (Fig. 4) these treatment conditions are an attempt to produce the least disruptive effect upon the integrity and viability of the red cell while at the same time provide for reasonable enzyme kinetics. We have found that under such conditions human red cells after as long as three hours of incubation with free enzyme or buffer alone show very slight hemolysis, on the order of 1-2%, and the enzyme-dextran conjugate even less; about 1%. Other experiments indicate that approximately 50% of the observed hemolysis is due to the end-over-end mixing procedure. Furthermore, fragility studies reveal that the 50% hemolysis concentrations of sodium chloride (cells from plasma = 0.46% sodium chloride; buffer incubated cells = 0.49%; enzyme treated cells = 0.48%; enzyme-dextran treated cells = 0.45%) are very similar, demonstrating that our treatment conditions do not produce any significant increase in susceptibility of these cells to osmotic shock and suggesting that we have not induced premature aging of our converted cells. This is further supported by microscopic examination of enzyme and enzyme-dextran treated cells which reveals them to be free of gross morphological abnormalities over 95% being discocytes with some spiculed forms also present.

In preparation for *in vivo* survival studies, gibbon type B red cells are being tested for changes in adenosine triphosphate (ATP) levels which appear to govern cell deformability and 2,3 diphosphoglyceric acid (2,3 DPG) levels which provide for normal oxygen binding and exchange. Our results to date indicate that under current buffer and incubation conditions there is essentially no change in ATP levels and about 90% retention of 2,3 DPG for up to 3 hours of incubation. Human cell values obtained for similar time treatments including incubation with enzyme are on the order of 90% for ATP as found for the gibbon but exhibit a range of from 60%-90% for 2,3-DPG, the lower value found when citrate is included in the treatment buffer. There are indications that the lower value can be increased to 80%-90% 2,3 DPG when a higher phosphate concentration is used in the recovery buffer. These preliminary metabolic studies which indicate retention of metabolic capabilities by treated cells will be confirmed and extended. And finally, using radioactive chromium of very high specific activity (Cr^{51}–1 mc/5 μg) we have developed a method for labeling small amounts of gibbon red blood cells (150 μl cells in total volume of 0.45 ml). These labeled cells can easily be detected when returned to the circulation (1.95×10^7 c.p.m. can be injected). Using such a procedure we plan to tag control and α-galactosidase treated cells and study their survival times *in vivo*.

Publications List

"The Preparation and Properties of An α-Galactosidase Immobilized on a Soluble Support", J.-Y. Kuo and J. Goldstein, Fed. Proc. 38 418 Abs. #998 (1979).